IVD

For Professional Use Only

GUIDELINES

to AmpliSens® *TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT PCR kit

for qualitative detection and differentiation of tick-borne infection pathogens: RNA of *TBEV – tick-borne encephalitis virus, Borrelia burgdorferi sl* – Ixodes tick-borne borreliosis (ITB) pathogen, *Ehrlichia chaffeensis* and *Ehrlichia muris* – human monocytic ehrlichiosis (HME) pathogens and DNA of *Anaplasma phagocytophilum* – human granulocytic anaplasmosis (HGA) pathogen in biological materials by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

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INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens**® ***TBEV, B.burgdorferi sl, A.phagocytophilum, E.chaffeensis / E.muris*-FRT** PCR kit for qualitative detection of RNA of *tick-borne encephalitis virus* (*TBEV), Borrelia burgdorferi sl* (Ixodes tick-borne borreliosis (ITB) pathogen), *Ehrlichia chaffeensis* and *Ehrlichia muris (*human monocytic ehrlichiosis (HME) pathogens) and DNA of *Anaplasma phagocytophilum (*human granulocytic anaplasmosis (HGA) pathogen) in biological materials (ticks, blood, cerebrospinal fluid, and autopsy material) by using real-time hybridization-fluorescence detection with

- Rotor-Gene 3000 (three and more channels) (Corbett Research, Australia),
- Rotor-Gene 6000 (five and six channels) (Corbett Research, Australia),
- Rotor-Gene Q (Qiagen, Germany),
- iCycler iQ, iQ5 (Bio-Rad, USA),
- Mx3000P (Stratagene, USA).

AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (Qiagen, Germany) INSTRUMENTS

Carry out sample pretreatment and reaction mixture preparation stages according to the instruction manual. Transparent flat-cap 0.2-ml PCR tubes and 0.1-ml tubes are recommended for use.

Place the tubes in the rotor so that the first tube is in well No. 1, insert the rotor in the reaction module, and secure the lid (the rotor cells are numbered and these numbers are used for the programming sample order).

The tubes with PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.* should be placed into the rotor first if both PCR-mixes-1 are used in the run

When working with the Rotor-Gene 3000 instrument, use the Rotor-Gene version 6 software. When working with the Rotor-Gene 6000 instrument, use the Rotor-Gene 6000 versions 1.7 (build 67) software or higher.

Hereinafter, all terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000.

Programming the Rotor-Gene 3000/6000, Rotor-Gene Q instrument

1. Click the ***New*** button in the main program menu.
2. In the opened window, select ***Advanced*** and click ***Dual Labeled Probe/Hydrolysis probes***. Activate the ***New*** button.
3. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***No Domed 0.2 ml Tubes/Locking ring attached*** for Rotor-Gene 6000 instrument. Click **Next**.

4. Reaction volume is ***25 µl.*** Enter an operator. Tick the ***15 µl oil layer volume*** option for Rotor-Gene 6000. Click ***Next***.
5. In the opened window click the ***Edit profile*** button and set the temperature profile of the experiment as follows:

Table 1

Amplification program for Rotor-Gene 3000/6000 and Rotor-Gene Q

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	Cycles
1	**95**	15 min		1
2	**95**	10 s		5
	60	30 s		
	72	15 s		
3	**95**	10 s		40
	56	30 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	15 s		

6. Click **OK**.
7. Click the ***Calibrate/Gain Optimization*** button in the ***New Run Wizard*** window.
8. Follow the steps:
   - perform calibration in the FAM/Green, JOE/Yellow, and ROX/Orange channels (click the ***Calibrate Acquiring/Optimize Acquiring*** button);
   - click the ***Perform Calibration Before 1st Acquisition/Perform Optimization Before 1st Acquisition*** button;
   - set channel calibration from 5Fl to 10Fl for all channels (the ***Edit*** button in the ***Auto gain calibration channel settings*** window). Click ***Close***.
9. Click ***Next***. Select the ***Start run*** button for amplification to run.
10. Name the experiment and save it to the disk (the results of the run will be automatically saved in this file).
11. Set the data in the table of samples (opens by default when run begins). Define names/numbers of clinical and control samples in the ***Name*** column. Enter ***None*** for empty wells.

Samples indicated as ***None*** will not be analyzed.

Data analysis

The results are interpreted by the Instrument software by the crossing (or not-crossing) of the fluorescence curve with the threshold line and are shown as the presence (or absence) of Ct (cycle threshold) in the results grid.

Data analysis of amplification in the **FAM/Green** channel.

1. Select the ***Analysis*** button in the main menu, select the ***Quantitation*** mode. Activate the ***Cycling A FAM/Cycling A Green*** button. Click ***Show***.
2. Cancel the automatic selection of the ***Threshold*** level.
3. The ***Dynamic tube*** and ***Slope Correct*** buttons should be activated in the ***Quantitation analysis*** window.
4. In the ***CT Calculation*** menu, set ***Threshold* = 0.03.**
5. Activate the ***More Settings*/*Outlier Removal*** button and enter ***NTC threshold*** level as **5** (5%) in the text field.
6. Ct value of each sample in the **FAM/Green** channel will appear in the results grid (***Quant. results*** window).

Data analysis of amplification in the **JOE/Yellow** channel.

1. Select the ***Analysis*** sign in the main menu, select the ***Quantitation*** mode. Activate the ***Cycling A JOE/Cycling A Yellow*** button. Click ***Show***.
2. Cancel the automatic selection of the ***Threshold*** level.
3. The ***Dynamic tube*** and ***Slope Correct*** buttons should be activated in the ***Quantitation analysis*** window.
4. In the ***CT Calculation*** menu, set ***Threshold* = 0.03.**
5. Activate the ***More Settings*/*Outlier Removal*** button and enter ***NTC threshold*** level as **5** (5%) in the text field.
6. Ct value of each sample in the **JOE/Yellow** channel will appear in the results grid (***Quant. results*** window).

Data analysis of amplification in the **ROX/Orange** channel.

1. Select the ***Analysis*** sign in the main menu, select the ***Quantitation*** mode. Activate the ***Cycling A ROX/Cycling A Orange*** button. Click ***Show***.
2. Cancel the automatic selection of the ***Threshold*** level.
3. The ***Dynamic tube*** and ***Slope Correct*** buttons should be activated in the ***Quantitation analysis*** window.
4. In the ***CT Calculation*** menu, set ***Threshold* = 0.03.**
5. Activate the ***More Settings*/*Outlier Removal*** button and enter ***NTC threshold*** level as **5** (5%) in the text field.
6. Ct value of each sample in the **JOE/Yellow** channel will appear in the results grid (***Quant. results*** window).

If fluorescence curves do not show exponential growth, set the ***NTC threshold*** as 10%

Result interpretation of the control samples

The result of the analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (Table 2). The results of positive and negative controls should not exceed Ct values specified in Table 2.

Table 2

Results for controls obtained with Rotor-Gene 3000/6000 and Rotor-Gene Q

Control	Stage for control	Ct value			Interpretation
		FAM/Green	JOE/Yellow	ROX/Orange	
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*					
		Detection of *TBEV*	**Detection of *A.phagocyto-philum***	**Detection of *E.chaffeensis / E.muris***	
C–	RNA/DNA extraction	Absent	Absent	Absent	OK
NCA	Amplification	Absent	Absent	Absent	OK
C+ ***TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI***	Amplification	<27	<27	<27	OK
PCR-mix-1-FRT *B.b. sl* / IC					
		Detection of IC	**Detection of *B.burgdorferi sl***	–	
C–	RNA/DNA extraction	<30	Absent	–	OK
NCA	Amplification	Absent	Absent	–	OK
C+ ***TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI***	Amplification	<27	<27	–	OK

Result interpretation for test samples

- *TBEV* cDNA is **detected** in a sample if its Ct value in the results grid in the FAM/Green channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 3.
- *A.phagocytophilum* DNA is **detected** in a sample if its Ct value in the results grid in the JOE/Yellow/HEX channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 3.
- *E.chaffeensis / E.muris* cDNA is **detected** in a sample if its Ct value in the results grid in the ROX/Orange channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 3.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if its Ct value in the results grid in the JOE/Yellow/HEX channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than

the Ct value specified in Table 3.

Moreover, the fluorescence curve of every studied sample should cross the threshold line at the exponential growth stage.

- cDNA/DNA of the above-mentioned microorganisms are not detected in a sample if the Ct value in FAM channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than the boundary Ct value specified in Table 3, while no Ct value is detected in the channel assigned for the specific pathogen.
- The result is **invalid** if the Ct value of a sample is absent in the channels for specific pathogen detection whereas Ct in the FAM/Green channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC)** is also absent or greater than the specified boundary Ct value. It is necessary to repeat the PCR test for such samples.

Table 3

Results for test samples obtained with Rotor-Gene 3000/6000 and Rotor-Gene Q

PCR-mix-1-FRT	**Signal in channel (Ct)**		
	FAM/Green	**HEX/Yellow**	**ROX/Orange**
TBEV, A.ph., E.ch. / E.m.	Detection of *TBEV*	Detection of *A.phagocytophilum*	Detection of *E.chaffeensis / E.muris*
	<38	<38	<38
***B.b. sl* / IC**	Detection of IC	Detection of *B.burgdorferi sl*	–
	<35	<38	–

Troubleshooting

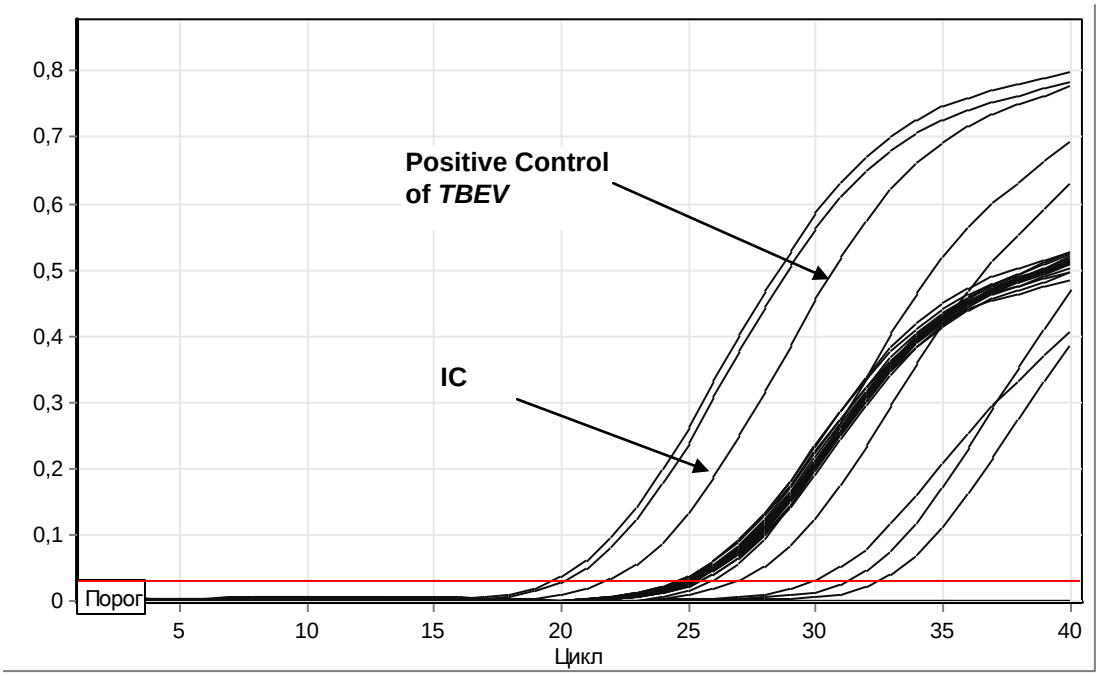
Results of analysis are not taken into account in the following cases:

- The samples (except for NCA) that show negative result in all channels should be analyzed once again (PCR with detection). If the same result is obtained in the second run, repeat the test starting from the extraction stage. For NCA, the Negative result detected in all channels is accepted as normal.
- If the Ct value of the Positive Control of amplification (C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ \mathrm{STI}}$) is absent or greater than the specified boundary Ct value in FAM, JOE, or ROX channels (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) or in the FAM and JOE channels (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**), PCR should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.
- If the Ct value of the Negative Control of extraction (C-) (in FAM, JOE, ROX channels with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and in the JOE channel with **PCR-mix-1-**

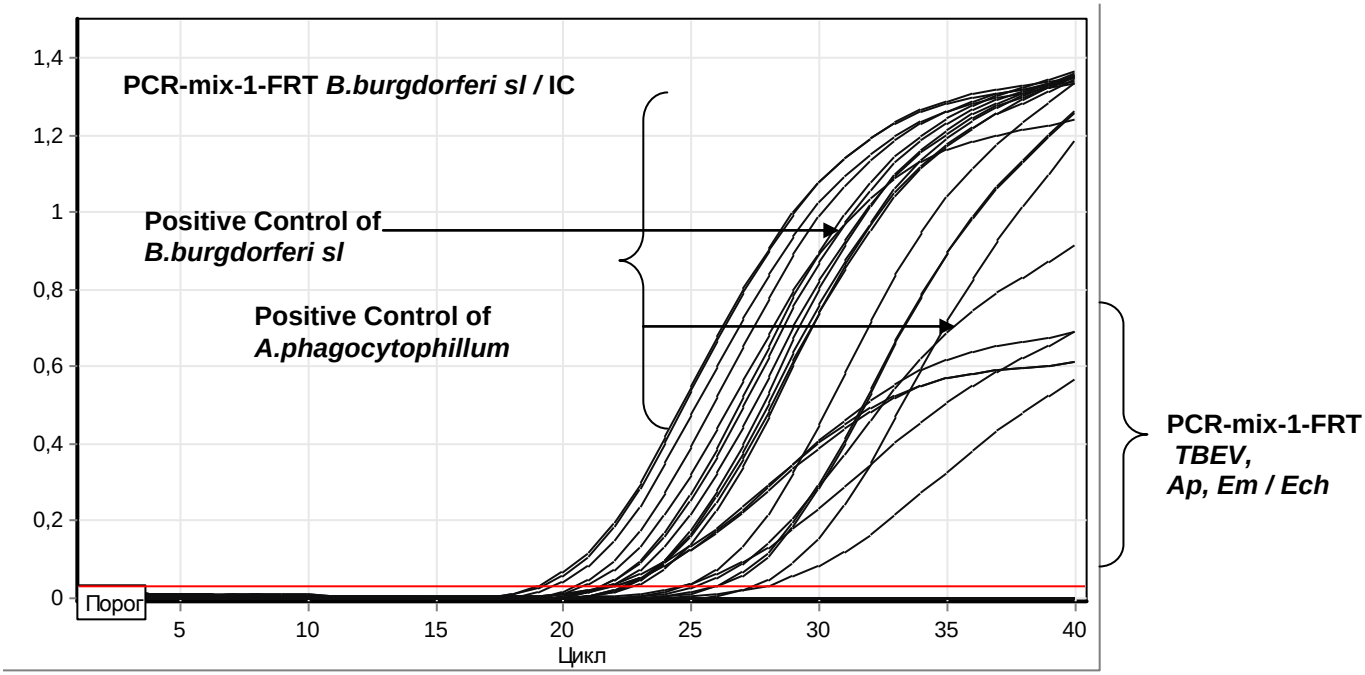
FRT *B.b. sl* / IC) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR should be repeated for all samples in which specific cDNA/ DNA was found in the appropriate channel.

Examples of obtained results

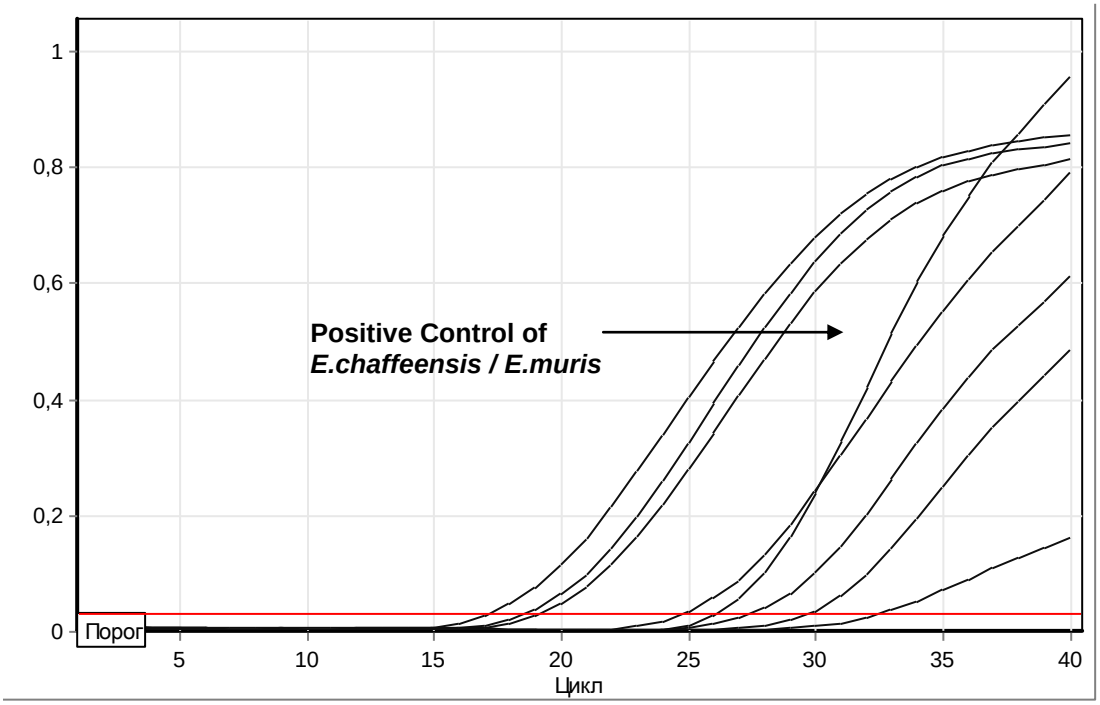
FAM channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch.* / *E.m.* and amplificaton of IC cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



JOE channel (amplification of *A.phagocytophilum* DNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.* and amplification of *B.burgdorferi sl* cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



ROX channel (amplification of *E.chaffeensis / E.muris* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*)



AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF iCycler iQ and iQ5 (Bio-Rad, USA) INSTRUMENTS

1. Switch on the instrument. The lamp should be warmed up for at least 30 min before the experiment starts.
2. Start the iCycler iQ/iQ5 program.
3. Define the plate setup, that is, a tube order in the reaction chamber and fluorescence signal detection in all tubes in FAM, JOE, and ROX channels if **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*** is used or in FAM and JOE channels if **PCR-mix-1-FRT *B.b. sl* / IC** is used.

   — **iCycler iQ 5 instrument**. In the ***Selected Plate Setup*** window of the ***Workshop*** module, press the ***Create New*** or ***Edit*** button. Edit the plate setup in the ***Whole Plate loading*** mode. Set ***Sample Volume*** as ***25 µl***, ***Seal Type*** as ***Domed Cap***, and ***Vessel Type*** as ***Tubes*****.** Press ***Save &Exit Plate Editing*****.**

   — **iCycler iQ instrument.** Edit the plate setup in the ***Edit Plate Setup*** window of the ***Workshop*** module. To do this, define the sample order in the ***Samples: Whole Plate Loading*** option and enter names of samples in the ***Sample Identifier*** window. In the ***Select and Load Fluorophores*** option, assign the detection of fluorescence signal in all tubes in FAM, JOE, and ROX channels if **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*** is used or in FAM and JOE channels if **PCR-mix-1-FRT *B.b. sl* / IC** is used.

   — To save the created plate setup, name it in the ***Plate Setup Filename*** window (use .pts file extension) and click the ***Save this plate setup*** button (at the top of the display). To edit a previously created plate setup, open ***View Plate Setup*** in the ***Library*** window and select the required plate setup (.pts file) and click the ***Edit*** button on the right. Save the edited file. Press the ***Run with selected protocol*** button to activate the created plate setup.
4. Set the amplification program (see Table 4).

Table 4

Amplification program for iCycler iQ and iQ5

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	Cycles
1	**95**	15 min		1
2	**95**	10 s		5
	60	35 s		
	72	15 s		
3	**95**	10 s		40
	56	35 s	**FAM, HEX, ROX**	
	72	15 s		

- **iCycler iQ5 instrument**. Click the ***Create New*** or ***Edit*** button in the ***Selected Protocol*** window of the ***Workshop*** module. Set the amplification parameters and click the ***Save&Exit Protocol Editing*** button to save the protocol. For further runs, a file with this program can be selected from the ***Protocol*** unit (all files are saved to the ***Users*** folder by default).
- **iCycler iQ instrument**. To set the amplification program, select the ***Edit Protocol*** option of the ***Workshop*** module. Enter the number of cycles, time, and temperature in the bottom window. Specify the signal acquiring in the window at the right as follows: Cycle 3 – Step. To save the protocol, name the file in the ***Protocol Filename*** window (use the .tmo file extention) and click the ***Save this protocol*** button (at the top of the display). For further runs, a file with this program can be selected from the ***View Protocol*** tab of the ***Library*** module. Press the ***Run with selected plate setup*** button to save and activate the created program.

5. Insert the prepared tubes into the reaction chamber in accordance with the specified tube order.

Prior to analysis, remove drops from tube walls, because drops falling during amplification cause signal errors and complicate data analysis. Do not turn strips/plate when placing it into the PCR instrument.

- **iCycler iQ5 instrument.** Ensure that the ***Selected Protocol*** and ***Selected Plate Setup*** are set correctly before starting the program. To start the program, click the ***Run*** button. Select the ***Use Persistent Well Factor*** option (set by default) for detection of the well factor. Make sure that amplification and calibration are performed with the same type of plastic consumables. Click the ***Begin Run*** button, name the experiment (result data will be automatically saved to this file) and click ***OK***. Select ***Seal Type – Domed cap***, and ***Vessel Type – Tubes***.
- **iCycler iQ instrument.** Ensure that the selected protocol and plate setup are set correctly in the ***Run Prep*** window. For determination of the well factor, select the ***Persistant Plate*** option in the ***Select well factor source*** line. Set the reaction mix volume as **25 µl** in the ***Sample Volume*** window. Click the ***Begin Run*** button, name the experiment (result data will be automatically saved in this file) and click ***OK***.

Proceed to analysis of results after the end of amplification run.

Analysis of results

The results are interpreted by the Instrument software by the crossing (or not-crossing) of the fluorescence curve with a threshold line and are shown as the presence (or absence) of Ct (threshold cycle) in the results grid. The fluorescence curve for the sample should contain a segment of a typical exponential growth (S-shape) of fluorescence and cross the

threshold line once.

Data processing

- **iCycler iQ** instrument. Activate the ***View Post-Run*** window in the ***Library*** module. Select the required file in the ***Data Files*** window and click the ***Analyze Data*** button. Select the desired channel in the ***PCR Quantification*** option of the ***Select a Reporter*** menu. Make sure that ***PCR Base Line Subtracted Curve Fit*** mode is activated (selected by default). In the ***Threshold Cycle Calculation*** menu indicate that the threshold is to be set manually and the base line is to be calculated automatically. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** submenu and select ***User Define*** in the ***Threshold Position*** submenu. To set the threshold line it should be move with the cursor while the left mouse button is pushed. When data obtained with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*** are processed, disable the wells that were analyzed with **PCR-mix-1-FRT *B.b. sl* / IC** in the ***Display wells*** menu. When data obtained with **PCR-mix-1-FRT *B.b. sl* / IC** are processed, disable the wells that were analyzed with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.***in the ***Display wells*** menu. Click the ***Recalculate Threshold Cycles*** button. Ct values will appear in the results grid.
- **iQ5** instrument. Select the required result data file (***Data File*** window of the ***Workshop*** module) and click the ***Analyz***e button. Select data obtained in the required channel in the window of the module. Make sure that the ***PCR Base Line Subtracted Curve Fit*** mode is activated (selected by default).

Analysis of amplification results obtained with PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m:*

***TBEV* cDNA**

1. Click the ***FAM*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select ***User Defined***, ***Select all***, and ***Edit Range*** in the ***Base Line Cycles*** menu and set ***Start Cycle=2, Ending Cycle=25***; select ***User Defined*** in the ***Crossing Threshold*** menu and set ***Threshold Position=200***. Click ***OK***.
4. Ct values will appear in the results grid (***Results*** window).

***A.phagocytophilum* DNA**

1. Click the ***JOE*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option using the right mouse button on the plot of the

fluorescence curves.

3. Set following parameters: select ***User Defined***, ***Select all***, and ***Edit Range*** in the ***Base Line Cycles*** menu and set ***Start Cycle=2, Ending Cycle=25***; select ***User Defined*** in the ***Crossing Threshold*** menu and set ***Threshold Position=100***. Click ***OK***.
4. Ct values will appear in the results grid (***Results*** window).

***E.muris, E.chaffensis* cDNA**

1. Click the ***ROX*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select ***User Defined***, ***Select all***, and ***Edit Range*** in the ***Base Line Cycles*** menu and set ***Start Cycle=2, Ending Cycle=25***; select ***User Defined*** in the ***Crossing Threshold*** menu and set ***Threshold Position=100***. Click ***OK***.
4. Ct values will appear in the results grid (***Results*** window).

Analysis of amplification results obtained with PCR-mix-1-FRT *B.b. sl* / IC*:*

Internal Control

1. Click the ***FAM*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select ***User Defined***, ***Select all***, and ***Edit Range*** in the ***Base Line Cycles*** menu and set ***Start Cycle=2, Ending Cycle=25***; select ***User Defined*** in the ***Crossing Threshold*** menu and set ***Threshold Position=50***. Click ***OK***.
4. Ct values will appear in the results grid (***Results*** window).

***B.burgdorferi sl* DNA**

1. Click the ***JOE*** button in the ***Data Analysis*** menu.
2. Select the ***Baseline Threshold*** option using the right mouse button on the plot of the fluorescence curves.
3. Set following parameters: select ***User Defined***, ***Select all***, and ***Edit Range*** in the ***Base Line Cycles*** menu and set ***Start Cycle=2, Ending Cycle=25***; select ***User Defined*** in the ***Crossing Threshold*** menu and set ***Threshold Position=100***. Click ***OK***.
4. Ct values will appear in the result grid (***Results*** window).

Result interpretation of the control samples

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct

(Table 5). Results of positive and negative controls should not exceed Ct values specified Table 5).

Table 5

Results for controls obtained with iCycler iQ and iQ5

Control	Stage for control	Ct value			Interpretation
		FAM	HEX	ROX	
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*					
		Detection of *TBEV*	**Detection of *A.phagocyto-philum***	**Detection of *E.chaffeensis / E.muris***	
C–	RNA/DNA extraction	Absent	Absent	Absent	OK
NCA	Amplification	Absent	Absent	Absent	OK
C+ ***TBEV, B.b. sl, A.ph., E.ch. / E.m.*** **/ STI**	Amplification	<30	<31	<30	OK
PCR-mix-1-FRT *B.b. sl* / IC					
		Detection of IC	**Detection of *B.burgdorferi sl***	–	
C–	RNA/DNA extraction	<33	Absent	–	OK
NCA	Amplification	Absent	Absent	–	OK
C+ ***TBEV, B.b. sl, A.ph., E.ch. / E.m.*** **/ STI**	Amplification	<30	<30	–	OK

Result interpretation of test samples

- *TBEV* cDNA is **detected** in a sample if its Ct value in the results grid in the FAM channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 6.
- *A.phagocytophilum* DNA is **detected** in a sample if its Ct value in the results grid in the HEX channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 6.
- *E.chaffeensis / E.muris* cDNA is **detected** in a sample if its Ct value in the results grid in the ROX channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 6.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if its Ct value in the results grid in the JOE/Yellow/HEX channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than the Ct value specified in Table 6.

Moreover, the fluorescence curve of each studied sample should cross the threshold line at the exponential growth stage.

- cDNA/DNA of the above-mentioned microorganisms are not detected in a sample if the Ct value detected in FAM channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than boundary Ct value specified in Table 6, while no Ct value is detected in the channel assigned for the specific pathogen.
- The result is **invalid** if Ct of a sample is absent in the channels for specific pathogen detection whereas in the FAM channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC)** Ct is also absent or greater than the specified boundary Ct value. Repeat the PCR test for such samples.

Table 6

Results for test samples obtained with iCycler iQ and iQ5

PCR-mix-1-FRT	**Signal in channel (Ct)**		
	FAM	**HEX**	**ROX**
TBEV, A.ph., E.ch. / E.m.	Detection of *TBEV*	Detection of *A.phagocytophilum*	Detection of *E.chaffeensis / E.muris*
	<39	<39	<39
***B.b. sl* / IC**	Detection of IC	Detection of *B.burgdorferi sl*	–
	<38	<39	–

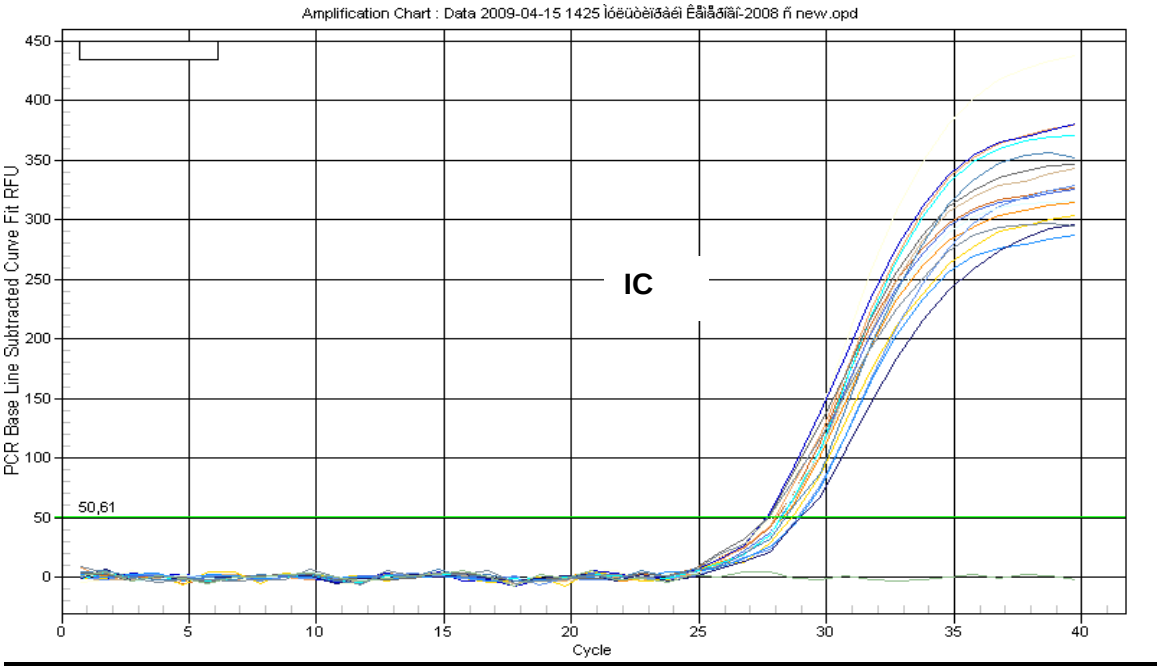
Troubleshooting

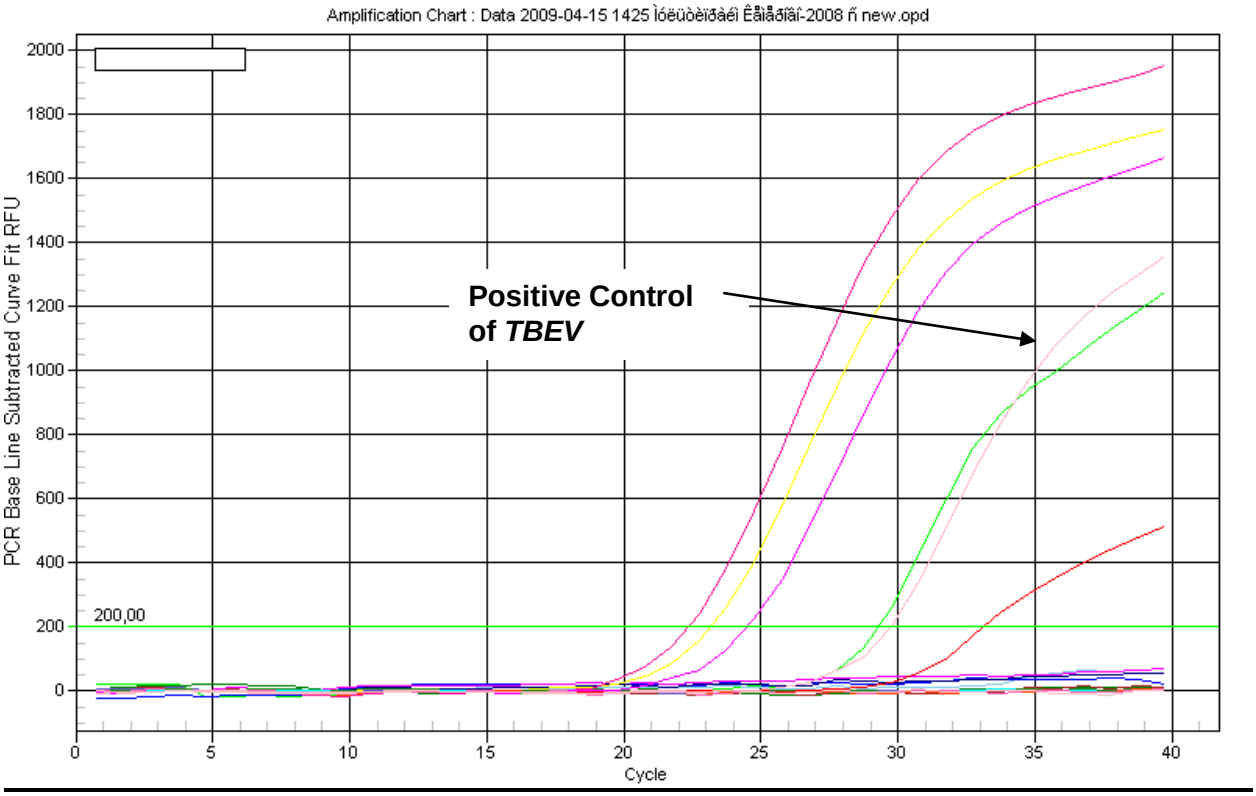
Results of analysis are not taken into account in the following cases:

- The samples (except for NCA) that show negative result in all channels should be analyzed once again (PCR with detection). If the same result is obtained in the second run, repeat the test starting from the extraction stage. For NCA, the Negative result detected in all channels is accepted as normal.
- If the Ct value of the Positive Control of amplification (C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ \mathrm{STI}}$) is absent or greater than the specified boundary Ct value in FAM, JOE, or ROX channels (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) or in the FAM and JOE channels (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**), PCR should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.
- If the Ct value of the Negative Control of extraction (C-) (in the FAM, JOE, ROX channels with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and in the JOE channel with **PCR-mix-1-FRT *B.b. sl* / IC**) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR should be repeated for all samples in which specific cDNA/ DNA was found in the appropriate channel.

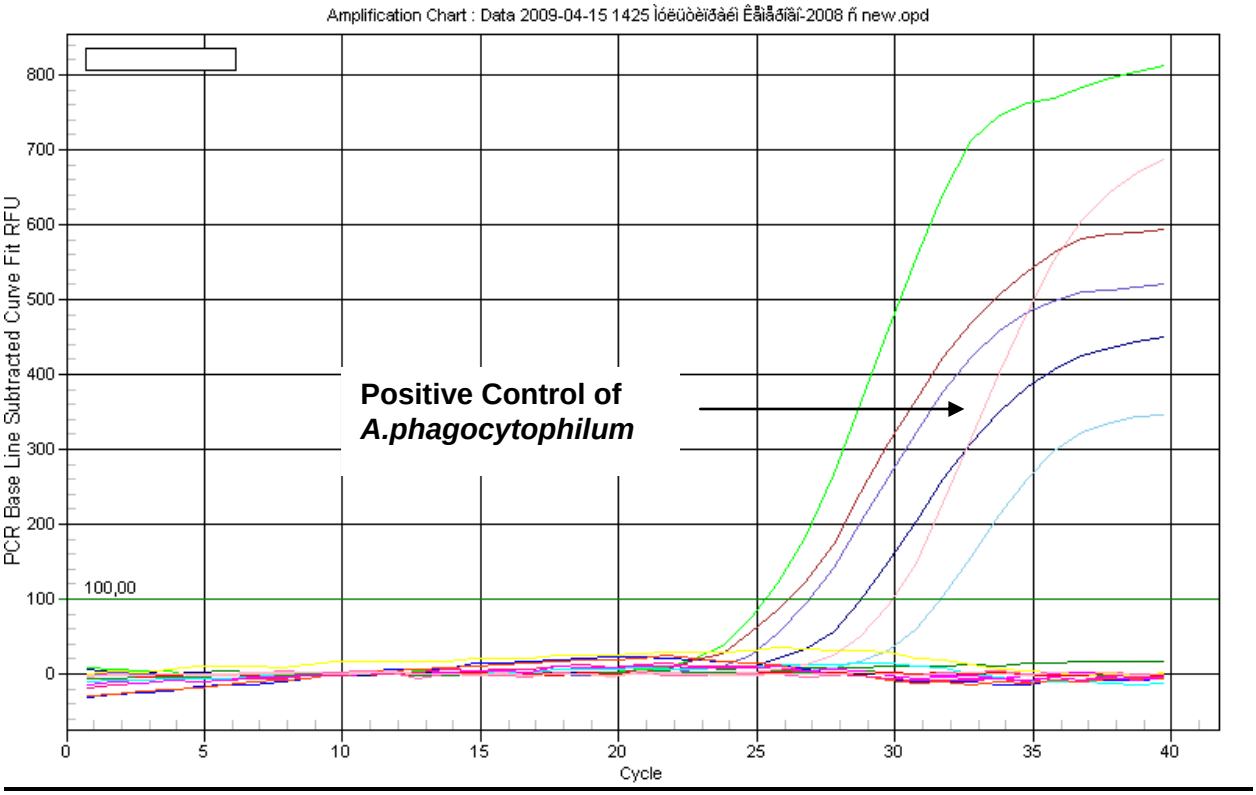
Examples of obtained results

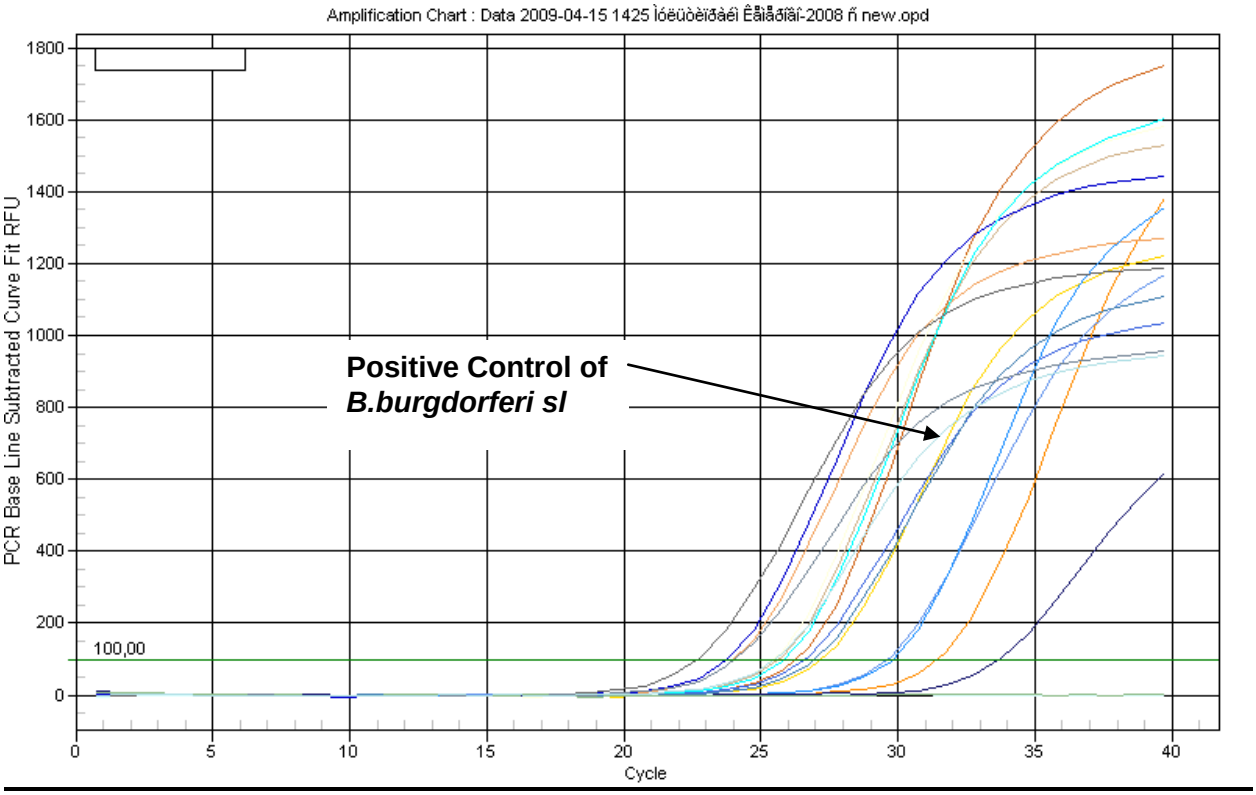
FAM channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch.* / *E.m.* and amplificaton of IC cDNA for PCR-mix-1-FRT *B.b. sl* / IC)


Amplification Chart : Data 2009-04-15 1425 Ìóëüòèïðàéí Êðàñíîäàð-2008 ñ new.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
IC
50,61

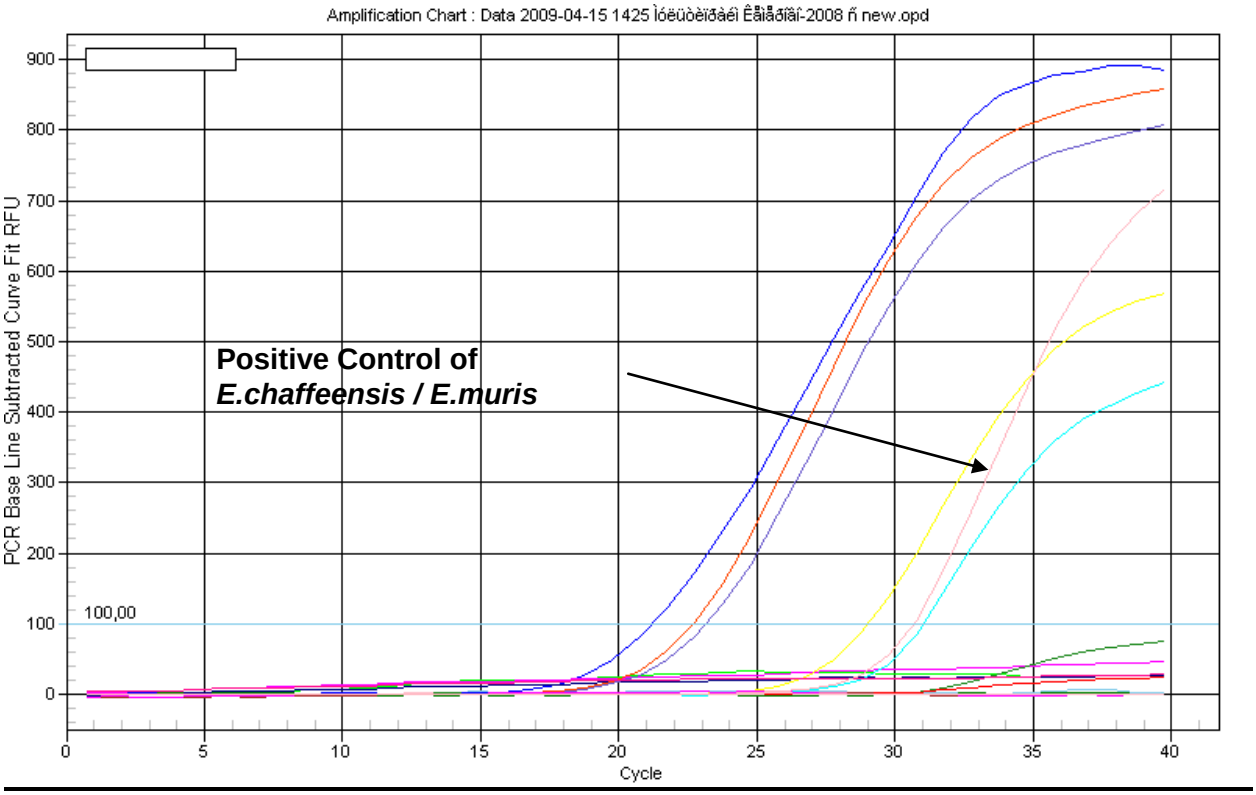


JOE channel (amplification of *A.phagocytophilum* DNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.* and amplification of *B.burgdorferi sl* cDNA for PCR-mix-1-FRT *B.b. sl* / IC)




Amplification Chart : Data 2009-04-15 1425 Ìóëüòèïðàêî Êåìåðîâî-2008 ñ new.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
100,00
Positive Control of
B.burgdorferi sl

ROX channel (amplification of *E.chaffeensis / E.muris* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*)


Amplification Chart : Data 2009-04-15 1425 Ìóëüòèïðàéì Êâàäðîâ-2008 ñ new.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
100,00
Positive Control of
E.chaffeensis / E.muris

AMPLIFICATION AND DATA ANALYSIS WITH THE USE OF Mx3000P (Stratagene, USA) INSTRUMENT

Carry out sample pretreatment and reaction mixture preparation stages according to the instruction manual. Program the instrument according to the user manual provided by the manufacturer.

1. Switch on the instrument. Start the ***Stratagene Mx3000P*** program.
2. Select ***Quantitative PCR (Multiple Standards)*** and check ***Turn lamp on for warm-up*** in the ***New Experiment Options*** window.

The lamp should be warmed up for at least 15 min before the experiment starts.

3. Place experimental tubes into the module and lock it.
4. Select ***Optics Configuration*** in th**e *Options*** menu. In the ***Dye Assignment*** window, set **FAM** next to the ***FAM filter set*** line, **JOE** next to the ***HEX/JOE filter set*** line, and **ROX** next to the ***ROX filter set*** line.
5. Set fluorescence detection parameters in the ***Plate Setup*** menu. To do this, select all cells with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E. m.*** test tubes and define them as ***Unknown*** in the ***Well type*** drop-down window. Select **FAM**, **JOE**, and **ROX** fluorophores in the ***Collect fluorescence data*** option. Then, select all cells with **PCR-mix-1-FRT *B.b. sl* / IC** test tubes and define them as ***Unknown*** in the ***Well type*** drop-down window. Select **FAM** and **JOE** fluorophores in the ***Collect fluorescence data*** option.
6. Enter the names of the test samples in the ***Well Information*** window.
7. In the ***Plate Setup*** menu, select all cells with the test tubes and select ***Unknown*** in the ***Well type*** drop-down window. Activate FAM, JOE, and ROX fluorophores in the ***Collect fluorescence data*** option.
8. Set the amplification program (see the table below) in ***Thermal Profile Setup***.

Table 7

Amplification program for Mx3000P instrument

Step	*Temperature, °C*	*Time*	*Fluorescent signal detection*	Cycles
1	**95**	15 min		1
2	**95**	10 s		5
	60	35 s		
	72	15 s		
3	**95**	10 s		40
	56	35 s	**FAM, JOE, ROX**	
	72	15 s		

9. Select ***Run*** in the menu. Ensure that amplification program is correct. Click ***Start***. Tick the ***Turn lamp off at end of run*** box. Save the experiment.

Analysis of results

Data processing

1. Open the saved data file and shift to ***Analysis*** mode.
2. Activate the ***Results*** window in the menu.
3. Select the ***Amplification plots*** line in the ***Area to analyze*** unit.
4. In the ***Threshold fluorescence*** unit set the threshold line at a level where fluorescence curves are linear. It is recommended to set the threshold level equal to **500** for all channels. Normally, the threshold line should cross only sigmoid curves of positive samples and controls and should not cross the base line. Otherwise, raise the threshold.
5. Select the ***Text report*** line in the ***Area to analyze*** unit.

Result interpretation for control samples

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (Table 8). Results of positive and negative controls should not exceed Ct values specified in Table 8)

Table 8

Results for controls obtained with Mx3000P

Control	Stage for control	Ct value			Interpretation
		FAM	JOE	ROX	
PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*					
		Detection of *TBEV*	**Detection of *A.phagocyto-philum***	**Detection of *E.chaffeensis / E.muris***	
C–	RNA/DNA extraction	Absent	Absent	Absent	OK
NCA	Amplification	Absent	Absent	Absent	OK
C+*TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI*	Amplification	<30	<31	<30	OK
PCR-mix-1-FRT *B.b. sl* / IC					
		Detection of IC	**Detection of *B.burgdorferi sl***	–	
C–	RNA/DNA extraction	<34	Absent	–	OK
NCA	Amplification	Absent	Absent	–	OK
C+*TBEV, B.b. sl, A.ph., E.ch. / E.m. / STI*	Amplification	<30	<30	–	OK

Result interpretation for test samples

- *TBEV* cDNA is **detected** in a sample if its Ct value in the results grid in the FAM channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 9.
- *A.phagocytophilum* DNA is **detected** in a sample if its Ct value in the results grid in the JOE channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 9.
- *E.chaffeensis* / *E.muris* cDNA is **detected** in a sample if its Ct value in the results grid in the ROX channel (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) is less than the Ct value specified in Table 9.
- *Borrelia burgdorferi sl.* cDNA is **detected** in a sample if its Ct value in the result grid in the JOE channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than the Ct value specified in Table 9.

Moreover, the fluorescence curve of each studied sample should cross the threshold line at the exponential growth stage.

- cDNA/DNA of the above-mentioned microorganisms are not detected in a sample if the Ct value detected in the FAM channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**) is less than boundary Ct value specified in Table 9, while no Ct value is detected in the channel assigned for the specific pathogen.
- The result is **invalid** if Ct of a sample is absent in the channels for specific pathogen detection whereas in the FAM channel (with the use of **PCR-mix-1-FRT *B.b. sl* / IC)** Ct is also absent or greater than the specified boundary Ct value. Repeat the PCR test for such samples.

Table 9

Results for test samples obtained with Mx3000P

PCR-mix-1-FRT	**Signal in channel (Ct)**		
	FAM	**HEX**	**ROX**
TBEV, A.ph., E.ch. / E.m.	Detection of *TBEV*	Detection of *A.phagocytophilum*	Detection of *E.chaffeensis* / *E.muris*
	<39	<39	<39
***B.b. sl* / IC**	Detection of IC	Detection of *B.burgdorferi sl*	–
	<38	<39	–

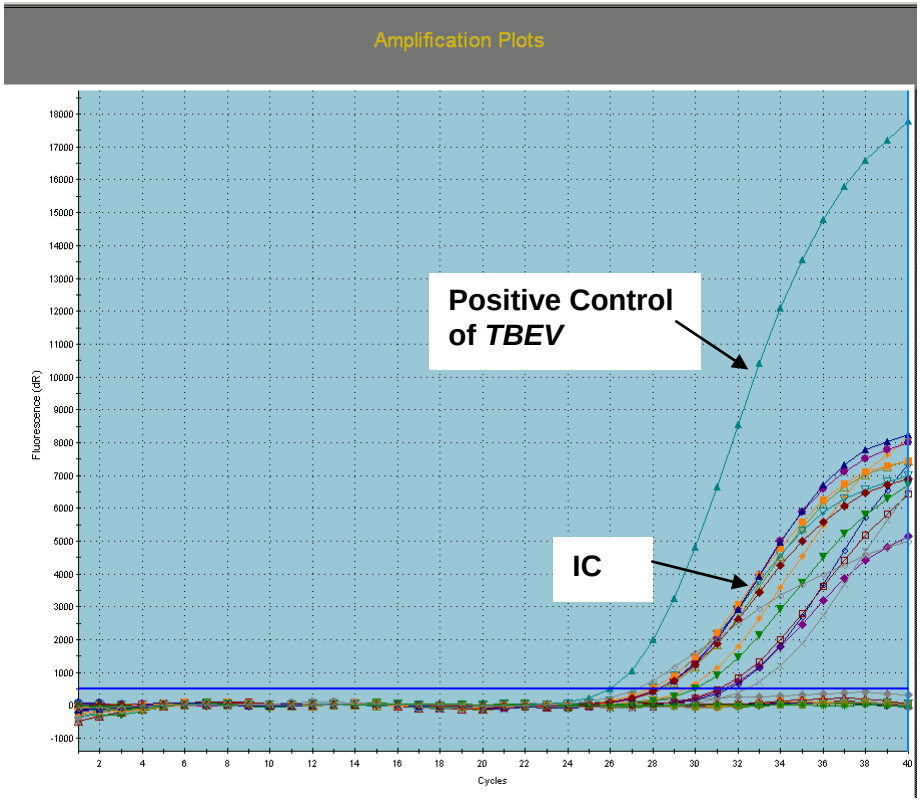
Troubleshooting

Results of analysis are not taken into account in the following cases:

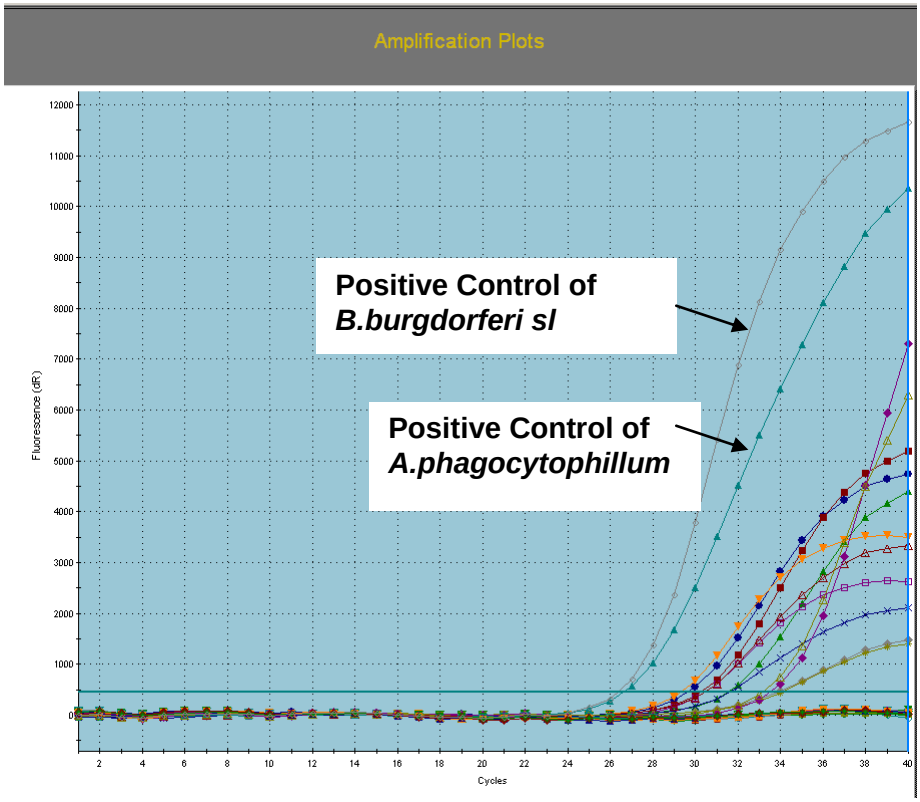
- The samples (except for NCA) that show negative result in all channels should be analyzed once again (PCR with detection). If the same result is obtained in the second run, repeat the test starting from the extraction stage. For NCA, the negative result detected in all channels is accepted as normal.
- If the Ct value of the Positive Control of amplification (C+$_{TBEV,\ B.b.\ sl,\ A.ph.,\ E.ch.\ /\ E.m.\ /\ STI}$) is absent or greater than the specified boundary Ct value in FAM, JOE, or ROX channels (with the use of **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.***) or in FAM and JOE channels (with the use of **PCR-mix-1-FRT *B.b. sl* / IC**), PCR should be repeated for all samples in which specific cDNA/DNA was not found in the appropriate channel.
- If the Ct value of the Negative Control of extraction (C-) (in FAM, JOE, ROX channels with **PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*** and in the JOE channel with **PCR-mix-1-FRT *B.b. sl* / IC**) and/or Negative Control of amplification (NCA) (in any channel) is detected in the results grid, PCR should be repeated for all samples in which specific cDNA/ DNA was found in the appropriate channel.

Examples of obtained results

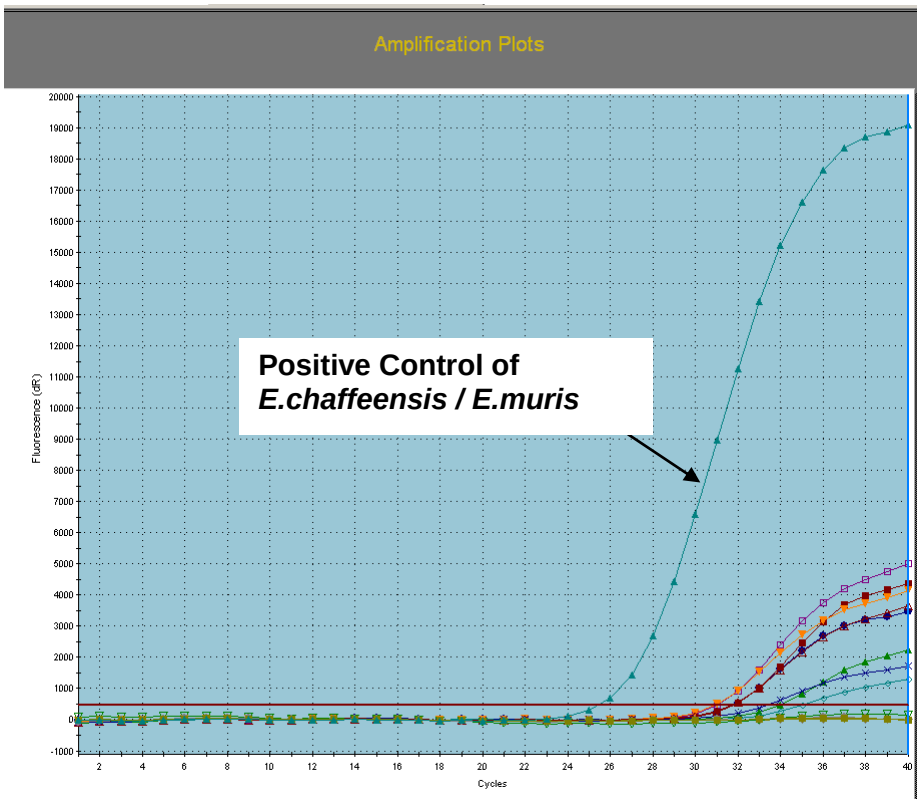
FAM channel (amplification of *TBEV* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.* and amplificaton of IC cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



JOE channel (amplification of *A.phagocytophilum* DNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.* and amplification of *B.burgdorferi sl* cDNA for PCR-mix-1-FRT *B.b. sl* / IC)



ROX channel (amplification of *E.chaffeensis / E.muris* cDNA for PCR-mix-1-FRT *TBEV, A.ph., E.ch. / E.m.*)



List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
29.06.11 LA	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
03.04.14 SA	Cover page	Address of European representative was added